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Malaria: **A sporozoite runs through it**

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A recent study reveals new insights into the development of *Plasmodium* sporozoites, the infectious agents of malaria. These findings may lead to changes in the approach to malaria vaccines and novel interpretations of the mechanisms of immunity to malaria.

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The infectious agent of malaria, the *Plasmodium falciparum* sporozoite, is injected into the skin by the mosquito as it probes for a blood vessel from which to feed. Few sporozoites are injected directly into the circulation via the mosquito bites. The sporozoite efficiently leaves the skin, localizes in the liver, and invades hepatocytes. One single *Plasmodium* sporozoite in one liver cell multiplies into tens of thousands of exoerythrocytic merozoites, each of which is able to invade a red blood cell, initiating the stage of the infection that causes the malarial disease. A percentage of asexual parasites convert to gametocytes, which are able to infect mosquitoes that transmit the infection to other humans. How the sporozoite travels from the skin to the liver, localizes to the liver, and invades hepatocytes are mysteries that are beginning to be elucidated. A recent study describes a new phase in this process in which sporozoites migrate through several cells, including hepatocytes, before settling into a hepatocyte for multiplication [1].

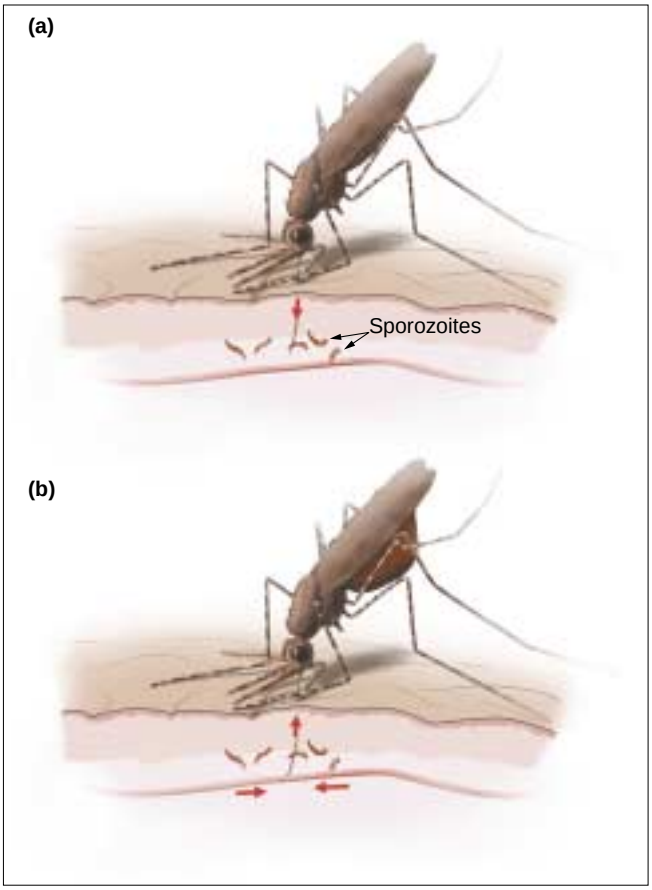
Malaria was described as a disease by Hippocrates in the fourth century BC, but the parasitic nature of the infection was not revealed until 1880 by Laveran. Ronald Ross, a British scientist working in India in 1897, then discovered that mosquitoes were vectors of malaria. He observed sporozoites of avian malaria within Culex mosquitoes and found that these sporozoites produced chicken infections; then workers in Italy showed the critical role of Anopheles mosquitoes in human malaria. Because malaria infects red blood cells, easily identified in stained blood films in patients, it was natural to assume that sporozoites directly invaded these cells. Schaudinn, in 1903, claimed he saw a sporozoite enter a red blood cell, a misconception that held up the pursuit of the events that take place before red blood cell invasion. Sporozoites of avian malaria were shown to multiply first in macrophages within the skin, then in other organs. The site for sporozoite development in primate malaria was unveiled much later from studies with monkey malaria. Shortt and Garnham [2] searched for the elusive exoerythrocytic merozoite (EE) forms in tissue sections from every organ of a monkey that had been bitten by thousands of *P. cynomolgi*-infected mosquitoes and then injected intravenously with tens of millions of sporozoites. Only liver cells were infected.

We shall briefly discuss the invasion of hepatocytes by sporozoites in malaria in a broader context of how parasites enter cells during different stages of their life cycle. All protists of the phylum Apicomplexa are defined by a set of organelles, called rhoptries and micronemes, at the apical end of these parasites. The phylum includes important pathogens of humans such as *Plasmodium* spp., *Toxoplasma gondii* and *Cryptosporidium parvum* as well as members exclusively parasitic in animals such as *Eimeria*, *Neospora*, and *Sarcocystis*. In *Plasmodium*, there are three invasive stages involving the apical organelles: ookinetes, which invade the mosquito midgut epithelium; sporozoites, which invade the mosquito salivary glands and host hepatocytes; and merozoites, which invade host red blood cells.

The first mechanism of invasion described was for the erythrocytic merozoites [3]. The merozoite contacts the red blood cell surface, reorients with its apical end against the cell, forms a junction with the cell, and pulls itself into the cell in a vacuole formed as the merozoite enters. The vacuole originates from the host plasma membrane, and its formation is presumably induced by rhoptry proteins [4]. Evidence for the critical nature of the junction derived first from studies of *P. knowlesi*, which was shown to require the expression of Duffy blood group determinants for both junction formation and invasion of red blood cells [5]. The parasite receptor that binds to the Duffy blood group determinants localizes in the micronemes [6], an organelle that also contains other receptors such as EBA-175, a Duffy-like molecule of *P. falciparum*, and the circumsporozoite protein of sporozoites.

Motility and cell invasion by Apicomplexan parasites are exquisitely sensitive to cytochalasins, implying that both processes are dependent on actin filaments in the parasite. The essential role of parasite but not host cell actin in powering motility and invasion was definitively shown in the case of *T. gondii* using a combination of host and parasite mutants that were resistant to cytochalasin D [7]. A variety of myosin isoforms has also been described in Apicomplexans, and indirect evidence indicates that they serve as motors for propelling cell locomotion and entry [8]. Kappe *et al.* [9] have identified the thrombospondinrelated anonymous protein (TRAP) as the critical membrane

Figure 1



Plasmodium-infected, female anopheline mosquitoes inject sporozoites through the proboscis (arrow) into the skin, together with saliva, which also contains vasodilators and anticoagulant molecules (**a**). The mosquitoes probe the skin to find and enter a blood vessel and immediately begin to suck blood (**b**). The arrows in (b) show the direction of blood flow from the vessel through the proboscis when a mosquito enters the vessel and sucks blood. Most sporozoites are deposited in the skin; few are inoculated directly into the circulation. Because the sporozoites have to traverse the endothelial cells to reach hepatocytes, an obligatory step for parasite development, these parasites must pass through other cells, as shown by Mota *et al.* [1]. (Image courtesy of D. Bliss.)

protein that interacts with the skeletal proteins in malarial sporozoites and in *T. gondii*.

The ookinete, the invasive stage in the mosquito midgut, traverses the peritrophic membrane in the mosquito midgut by secreting chitinase. It then enters the apical side of epithelial cells, and leaves the basal side to form an oocyst — the extracellular, proliferative form of the parasite in which sporozoites are formed. Despite the fact that it has been over 100 years since Ross described the invasion of epithelial cells, there remains controversy as to how the ookinetes invade and whether they invade a special subpopulation of epithelial cells [10,11]. Although invasion is critical to move the ookinetes into the next compartment for their development, it is in no way critical for signaling development. This stage of the invasion process can be bypassed, because ookinetes inoculated directly into the hemolymph develop into sporozoites and ookinetes can also develop into sporozoites *in vitro* [12].

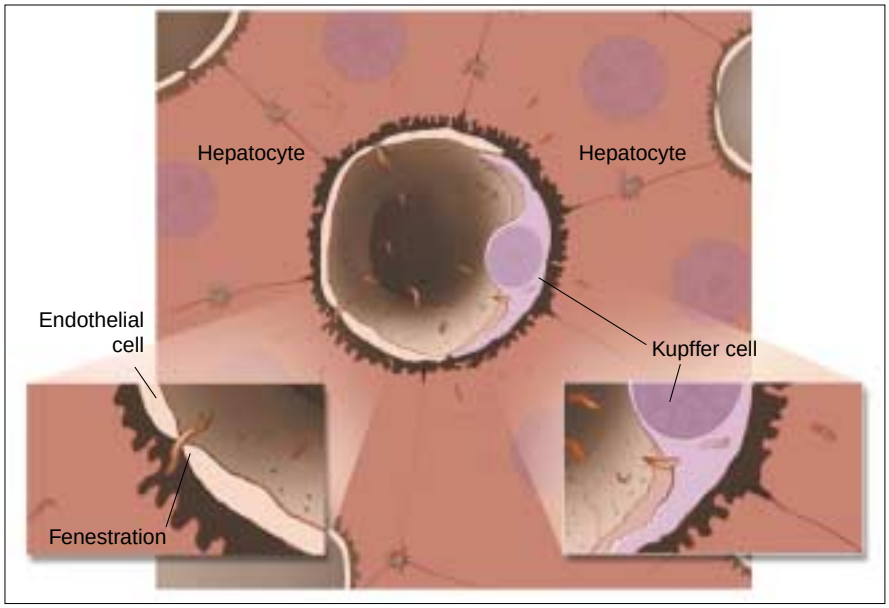
Sporozoites released from the oocyst in the mosquito hemocele must invade the salivary glands to reach the next host through a mosquito bite. Binding to specific receptors on the salivary glands seems necessary, because antisporozoite antibodies inhibit salivary gland invasion [13]. Sporozoites invade the salivary gland epithelial cells, apical end first, within a vacuolar membrane and appear to have a junction with the epithelial cell membrane at one end [14]. The vacuolar membrane is then rapidly lysed so that the sporozoites lie free within the cytoplasm.

Parasites delivered by mosquito bites are usually not injected directly into the circulation (Figure 1). As the mosquito searches for a blood vessel, it releases various vasodilators to increase its chance of finding a vessel; sporozoites, if present, are deposited in the skin as the mosquito hunts for a vessel [15]. When the mosquito enters a blood vessel, it ingests blood, and no longer releases saliva or sporozoites. Mice bitten by mosquitoes on the ears are infected less frequently if the site is excised within five minutes after feeding [16], providing further evidence that sporozoites are not released after the mosquito enters a vessel. How sporozoites leave the skin is unknown. The recent study by Mota *et al.* [1] demonstrates that sporozoites can travel through an epithelial layer *in vitro*, a mechanism that may permit the passage of sporozoites through vessels in the skin.

After sporozoites leave the skin and enter the circulation, they efficiently localize to the liver and invade hepatocytes. The efficiency of liver infection in the natural host can be high, as is the case for *P. berghei* sporozoites in the tree rat; the efficiency of liver infection by these sporozoites in mice, however, is quite different (50% and 1–3%, respectively) [17]. This efficiency difference was found to result from a failure of development of sporozoites in mouse hepatocytes, not a failure of infection [18]. Mice, although not the natural host for *P. berghei* and therefore less efficient, are used extensively for vaccination experiments. The sporozoite binds to specific receptors on liver cells that include glycoaminoglycan chains of heparan sulfate proteoglycans [19,20]. The co-receptor on sporozoites involves, in part, the thrombospondin domains on the circumsporozoite protein [21] and on TRAP [22]. The thrombospondin domain binds specifically to heparan sulfate proteoglycans on hepatocytes in the region in apposition to sinusoidal endothelia and Kupffer cells (see Figure 2 for diagram of liver structure).

Figure 2

Sporozoites, as they circulate through the sinusoids of the liver, must cross to the hepatocytes where they undergo development. It is unknown whether they pass through the Kupffer cells within a vacuole or through the fenestrations in the endothelia. (Image courtesy of D. Bliss.)



How then does the sporozoite reach hepatocytes? Do sporozoites pass through fenestrations in the endothelium in portal veins, or do they enter the space of Disse by traversing Kupffer cells? The rapidity with which sporozoites that are inoculated intravenously by syringe can enter hepatocytes might argue that they invade directly by migrating through the endothelial fenestrations that may expose the receptor-laden hepatocytes to the portal circulation (Figure 2). This passage through fenestrations, however, has never been observed.

Meis *et al.* [23] observed sporozoites passing through Kupffer cells in a vacuole, passing into the space of Disse. Recent work by Pradel and Frevert [24] supports the notion that sporozoites use Kupffer cells as a gate to the liver: the parasites bind to and actively invade Kupffer cells, but not sinusoidal endothelia *in vitro*. Upon invasion, sporozoites enter a vacuole in Kupffer cells that does not fuse with lysosomes, thus explaining why they are not being degraded. If this is the mechanism for entering the liver, how does the sporozoite recognize the Kupffer cells to reach the hepatocytes efficiently? Notably, avian and reptilian malaria species develop inside Kupffer cells, so that, conceivably, the mammalian species may have preserved this recognition mechanism. One possible model is that mammalian malaria sporozoites are initially arrested in the sinusoid by binding to the liver- specific heparan sulfate proteoglycans protruding into the sinusoidal lumen through the endothelial fenestration and glide with the bloodstream towards the next Kupffer cell, which they traverse to obtain access to the space of Disse.

Mota *et al.*, in the recent *Science* paper [1], address the mechanism of invasion of hepatocytes. They observed invasion *in vitro* of hepatoma cells and noted that sporozoites passed through the cells in a similar manner as that described by Vanderberg *et al.* [25]. The use of a marker for accessibility of large molecules (fluorescent dextran) to the cytoplasm and markers for the plasma membrane demonstrated that sporozoites in cells with dextran in the cytoplasm were not surrounded by the host plasma membrane. In cells surrounded by the host plasma membrane viewed 24 hours later, no dextran was seen spread through the cytoplasm. This indicated that sporozoites pass through many cells without having entered into a vacuole. In a few cells, sporozoites had invaded within a vacuole, and these were the only sporozoites that developed into EE forms, the multiplying parasite. Mota *et al.* [1] injected fluorescent dextran with the sporozoites *in vivo* and noted fluorescent dextran in the liver cells near the developing EE forms. They concluded that the parasite first passed through a number of hepatocytes before invading another hepatocyte by a vacuolar mechanism that led to EE formation.

What is the meaning of passing through cells outside of a vacuole before invading within a vacuole to develop into EE forms, and how does this invasion within a vacuole occur? One possibility is that the parasite surface changes to expose other receptors that are critical for invasion. In the case of *P. vivax* merozoites, the parasite only invades Duffy-positive reticulocytes. Invasion requires two different co-receptors — one for Duffy-positive cells and one for reticulocytes. Is it possible that, for *Plasmodium* sporozoites,

one receptor brings the parasite to the liver, and two receptors allow invasion within a vacuole? Is this invasion within a vacuole determined by a moving junction similar to the events in the red blood cell and what appears to happen within the salivary glands? The mystery still remains why the parasite needs to invade hepatocytes by one method before it can have a productive infection that leads to EE forms.

These critical details in parasite invasion remain to be determined in the many phases of the parasite life cycle and may play an important role in vaccine design to block invasion. For example, at least in the mouse model, high doses of passively transferred anti-circumsporozoite antibodies will block infection by sporozoites inoculated intravenously but not through mosquito bites [26]. Protection against mosquito challenge was shown, however, when high anti-circumsporozoite antibodies were induced by vaccination with the hybrid of circumsporozoite repeats [27] or the circumsporozoite protein [28] and the hepatitis B virus nucleocapsid antigen. Studies of the normal physiology of sporozoites in the skin, such as the one by Mota *et al.* [1], will inform the design of sporozoite vaccine studies that may be more relevant to protection from natural mosquito challenge.

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